

# Modular pathways for editing non-cognate amino acids by human cytoplasmic leucyl-tRNA synthetase

Xin Chen<sup>1</sup>, Jing-Jing Ma<sup>1</sup>, Min Tan<sup>1</sup>, Peng Yao<sup>1</sup>, Qing-Hua Hu<sup>1</sup>, Gilbert Eriani<sup>2</sup> and En-Duo Wang<sup>1,\*</sup>

<sup>1</sup>State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, The Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China and <sup>2</sup>Architecture et Réactivité de l'ARN, Institut de Biologie Moléculaire et Cellulaire du CNRS, Université de Strasbourg, 15 rue René Descartes 67084 Strasbourg, France

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## ABSTRACT

To prevent potential errors in protein synthesis, some aminoacyl-transfer RNA (tRNA) synthetases have evolved editing mechanisms to hydrolyze misactivated amino acids (pre-transfer editing) or misacylated tRNAs (post-transfer editing). Class Ia leucyl-tRNA synthetase (LeuRS) may misactivate various natural and non-protein amino acids and then mischarge tRNA<sup>Leu</sup>. It is known that the fidelity of prokaryotic LeuRS depends on multiple editing pathways to clear the incorrect intermediates and products in the every step of aminoacylation reaction. Here, we obtained human cytoplasmic LeuRS (hcLeuRS) and tRNA<sup>Leu</sup> (hctRNA<sup>Leu</sup>) with high activity from *Escherichia coli* overproducing strains to study the synthetic and editing properties of the enzyme. We revealed that hcLeuRS could adjust its editing strategy against different non-cognate amino acids. HcLeuRS edits norvaline predominantly by post-transfer editing; however, it uses mainly pre-transfer editing to edit  $\alpha$ -amino butyrate, although both amino acids can be charged to tRNA<sup>Leu</sup>. Post-transfer editing as a final checkpoint of the reaction was very important to prevent mis-incorporation *in vitro*. These results provide insight into the modular editing pathways created to prevent genetic code ambiguity by evolution.

## INTRODUCTION

The aminoacyl-transfer RNA (tRNA) synthetases (aaRSs) catalyze the activation of their cognate amino acids and transfer them to the appropriate tRNA molecules (1). Based on structural features, aaRSs can be divided into two classes, I and II (2). Leucyl-tRNA synthetase (LeuRS) falls into the first category, which is characterized by the HIGH and KMSK motifs. Structurally, all LeuRSs contain a bipartite Rossmann-fold catalytic domain (3), a large insertion domain called connective peptide 1 (CP1) within the catalytic domain (4), and a tRNA anticodon-binding domain close to the C-terminus (5). Furthermore, in the cytosol of higher eukaryotic cells, human cytoplasmic LeuRS (hcLeuRS) exists as a component of the multi-aminoacyl-tRNA synthetase complex (MSC), which consists of eight aaRSs and three additional non-synthetase accessory proteins (6). Similar to other eukaryotic aaRSs (7), LeuRS has been linked to human diseases. HcLeuRS is potentially implicated in lung tumorigenesis (8), and the mitochondrial LeuRS may be involved in diabetes (9).

The fidelity of aaRSs is sometimes threatened by a limited number of non-cognate standard amino acids. For example, isoleucyl-tRNA synthetase (IleRS) must distinguish between isoleucine and valine, which differ by a single missing methyl group (10,11). Valyl-tRNA synthetase (ValRS) also activates threonine, which has a hydroxyl group that is isosteric to the methyl moiety in valine (12). Leucyl-tRNA synthetase (LeuRS), which is homologous to IleRS and ValRS, misactivates a wide

\*To whom correspondence should be addressed. Tel: +86 21 5492 1241; Fax: +86 21 5492 1011; Email: edwang@sibs.ac.cn; wed@server.shnc.ac.cn

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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array of amino acids *in vitro* (13,14) as well as some non-standard amino acids (13). To clear these mistakes and maintain the fidelity of protein synthesis (15), some aaRSs have evolved editing mechanisms to hydrolyze misactivated amino acids (pre-transfer editing) or misacylated tRNAs (post-transfer editing). In addition, the elimination of a non-cognate aminoacyl-adenylate may occur through its preferential release and subsequent hydrolysis in solution (kinetic proofreading) (16). For example, IleRS performs pre- and post-transfer editing reactions but prefers the pre-transfer pathway by ~80–90% (17). ValRS rejects threonine mainly by the post-transfer pathway (12); however, whether or not ValRS catalyzes via pre-transfer editing is still unknown. Recent studies showed that LeuRS performs both pre- and post-transfer editings, and the *Escherichia coli* LeuRS favors post-transfer editing, whereas the *Aquifex aeolicus* enzyme favors pre-transfer editing (18,19). Class II aaRSs such as prolyl-tRNA synthetase (ProRS) (20), alanyl-tRNA synthetase (AlaRS) (21) and phenylalanyl-tRNA synthetase (PheRS) (22) all exhibit both pre- and post-transfer editing, whereas threonyl-tRNA synthetase (ThrRS) relies only on post-transfer editing (23), while seryl-tRNA synthetase (SerRS) performs only pre-transfer editing (24).

LeuRS, IleRS and ValRS form a subgroup of related synthetases with high sequence and structural homology (5). For post-transfer editing, a model was postulated wherein the flexible 3'-end of a misacylated tRNA is translocated from the aminoacylation active site to the hydrolytic editing site (25,26). The non-cognate aminoacyl-adenylates can be hydrolyzed via tRNA-dependent (10,21,27) or tRNA-independent pathways by LeuRS and other aaRSs (24,28). One possible model for tRNA-dependent pre-transfer editing has been proposed for IleRS and ValRS in which an initial post-transfer editing step is used to trigger a conformational change to an editing active conformation that can then perform pre-transfer editing in the CP1 domain (29). This model would require a migration of the adenylate from the synthetic site to the editing site, which are separated by ~30 Å. However, there is no apparent channel between the two sites, which could serve to prevent dissociation of the adenylate from the surface of the enzyme during translocation. On the other hand, several recent studies have shown that tRNA-dependent pre-transfer editing may occur in the synthetic active site. Class II ProRS and SerRS edit non-cognate aminoacyl-adenylates in their synthetic active site in a tRNA-independent way (24,30). For the class I LeuRS, the location of a tRNA-dependent pre-transfer editing activity within the synthetic site was proposed (18). It was also shown that class I GlnRS, which lacks an editing domain and any known editing activity, is able to catalyze an editing-like reaction in its synthetic active site (31).

Human cytoplasmic LeuRS (hcLeuRS) has been identified as a component of the multi-aminoacyl-tRNA synthetase complex (MSC) (32). It has been shown that the C-terminal appended domain of hcLeuRS is crucial for its interaction with other components of the MSC (33). More recently, the editing properties of hcLeuRS

were reported (34). Substitution of the crucial threonine residue from the CP1 editing domain did not induce the usual discriminating effect on the non-cognate amino acids, suggesting that the architecture of the editing site is different from that of other characterized LeuRSs (34). In the present study, recombinant human cytoplasmic tRNA<sup>Leu</sup> (hctRNA<sup>Leu</sup>) and hcLeuRS were over-expressed and purified from their *E. coli* transformants, separately. With the purified hctRNA<sup>Leu</sup> as substrate, hcLeuRS exhibited much higher aminoacylation and editing activities than those measured previously with tRNA transcript. We found that hcLeuRS can misactivate several Leu analogs, including norvaline (Nva),  $\alpha$ -amino butyrate (ABA), Met and Ile. We also show that hcLeuRS can charge hctRNA<sup>Leu</sup> with these amino acids and excluded the incorrect products by multiple editing pathways. These results showed that the human enzyme edits Nva essentially by the post-transfer editing pathway occurring in the CP1 domain. In contrast, editing of mis-activated ABA mainly occurred at the pre-transfer stage, suggesting the mechanical differences between editing pathways confers an advantage for effective proofreading.

## MATERIALS AND METHODS

### Materials

L-leucine, L-isoleucine, L-valine, L-methionine, L-norvaline,  $\alpha$ -amino butyric acid (ABA), 5'-GMP, ATP, GTP, CTP, UTP, tetrasodium pyrophosphate, and inorganic pyrophosphatase were purchased from Sigma (St. Louis, MO, USA). L-[<sup>3</sup>H]leucine and L-[<sup>3</sup>H]methionine (1 mCi/ml) were obtained from Perkin Elmer Life Sciences (Boston, MA, USA). T4 polynucleotide kinase, T4 DNA ligase, RNasin (ribonuclease inhibitor), isopropyl  $\beta$ -D-thiogalactoside (IPTG) and all restriction endonucleases were obtained from Sangon Co. (Shanghai, China). AN2690 was obtained from Milestone Pharmtech USA Inc. GF/C and DE-81 filters were obtained from the Whatman Co. (Dassel, Germany). *E. coli* strain Rosetta<sup>TM</sup> 2 (DE3) was purchased from Novagen, EMD Biosciences Inc. (Darmstadt, Germany). Plasmid pTrec99B, the gene encoding tRNA nucleotidyltransferase and *E. coli* strain MT102 were from the Institut de Biologie Moléculaire et Cellulaire du CNRS (Strasbourg, France). The tRNA nucleotidyltransferase was purified by the method described earlier (35).

### Enzymes and tRNA preparations

The gene encoding hcLeuRS was PCR-amplified from pFastBac1H-*hcLeuS* (33) and cloned between the NdeI and SalI sites of pET22b(+). For overexpression, *E. coli* strain Rosetta<sup>TM</sup> 2 (DE3) was used. Typically, 500 ml of 2 × YT medium supplemented with 60  $\mu$ g/ml chloramphenicol, 100  $\mu$ g/ml ampicillin were inoculated and incubated at 37°C until IPTG addition (200  $\mu$ M). Induction was performed at 20°C for 6 h. The purification of hcLeuRS on Ni<sup>2+</sup>-NTA Superflow column (Qiagen Inc., Germany) was carried out as described earlier (33).

hctRNA<sup>Leu</sup> (in this study refers to hctRNA<sup>Leu</sup><sub>CAG</sub> isoacceptor) was either extracted from cells or prepared

by *in vitro* transcription. The overexpressed hctRNA<sup>Leu</sup> was purified from *E. coli* cells (strain MT102) containing the gene encoding hctRNA<sup>Leu</sup> as described earlier (36,37). After incubation with IPTG at 37°C for 15 h, the harvested cells were extracted by phenol and the nucleic acid fraction was collected followed by loading onto a DEAE-Sepharose CL-6B column (2 × 20 cm). The column was eluted with a linear NaCl gradient, while the highest peak containing the tRNA<sup>Leu</sup> was collected and precipitated by ethanol. The tRNA sample from DEAE-Sepharose was further applied to a 25 ml C4 reversed-phase HPLC column (1 × 15 cm) equilibrated previously with buffer A (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 M NaHCO<sub>2</sub>, 8 mM MgCl<sub>2</sub>, pH 5.5). The retained material was eluted with programmed gradients of buffer A and B (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 10% ethanol, pH 5.5) and recovered by ethanol precipitation. *In vitro* transcription of hctRNA<sup>Leu</sup> and subsequent refolding of tRNA were performed as described earlier (33). The tRNA concentration was determined by UV absorbance at 260 nm and the extinction coefficient was calculated from the sequence of each tRNA (38).

### Amino acid activation

For hcLeuRS, the ATP-PPi exchange reaction was performed at 37°C in a reaction mixture containing 2 mM [<sup>32</sup>P]pyrophosphate (PPi) 15 cpm/pmol, 100 mM HEPES-KOH (pH 7.6), 6 mM MgCl<sub>2</sub>, 2 mM KF, 2 mM ATP, various concentrations of leucine (or Nva, or ABA, or Ile or Met) and 80 nM of hcLeuRS (37).

### AMP formation

For hcLeuRS, AMP formation was measured in reaction mixtures containing 1 mM spermine, 50 mM HEPES-KOH (pH 7.6), 25 mM KCl, 6 mM MgCl<sub>2</sub>, 5 mM DTT, 5 U/ml PPase, 3 mM ATP, 20 nM [ $\alpha$ -<sup>32</sup>P]ATP (3000 Ci/mmol, Perkin Elmer) and 2 mM Leu, or 10 mM Nva or 15 mM ABA, with or without 5  $\mu$ M of overproduced tRNA<sup>Leu</sup>. Reactions were incubated at 37°C and initiated by 1  $\mu$ M hcLeuRS, or the corresponding mutants in the presence of the amino acid. Aliquots (1.5  $\mu$ l) were quenched in 6  $\mu$ l of 200 mM sodium acetate (pH 5.0). Quenched aliquots (1.0  $\mu$ l each) were spotted in duplicate on polyethyleneimine cellulose plates (Merck) pre-washed with water. Separation of AA-[<sup>32</sup>P]AMP, [<sup>32</sup>P]AMP and [<sup>32</sup>P]ATP was performed by developing TLC plates in 0.1 M ammonium acetate and 5% acetic acid (39). The plates were visualized by phosphor imaging and the data were analyzed using ImageQuant 5.2 software (GE Healthcare). The gray densities of [<sup>32</sup>P]AMP and AA-[<sup>32</sup>P]AMP spots were compared to the gray density of a known [<sup>32</sup>P]ATP concentration.

### Aminoacylation and deacylation

The aminoacylation assay was carried out in a buffer containing 1 mM spermine, 50 mM HEPES-KOH (pH 7.6), 25 mM KCl, 6 mM MgCl<sub>2</sub>, 20  $\mu$ M [<sup>3</sup>H]Leu or 75  $\mu$ M [<sup>3</sup>H]Met, 4 mM ATP, 10  $\mu$ M tRNA<sup>Leu</sup> and 25 nM hcLeuRS or 1.5  $\mu$ M hcLeuRS-D399A mutant at 37°C.

Deacylation assays were performed at 37°C in reactions identical to the aminoacylation condition except that the amino acid and tRNA were replaced by 1  $\mu$ M [<sup>3</sup>H]Met-tRNA<sup>Leu</sup>.

To determine the misacylation of non-cognate amino acids by hcLeuRS mutant, reactions were performed as described earlier (40,41). HctRNA<sup>Leu</sup> was labeled at the 3'-terminal internucleotide linkage using the exchange reaction catalyzed by tRNA nucleotidyltransferase. Reactions contained 50 mM Tris-HCl buffer (pH 8.0), 12 mM MgCl<sub>2</sub>, 5 mM DTT, 3  $\mu$ M hctRNA<sup>Leu</sup>, 0.5  $\mu$ M [ $\alpha$ -<sup>32</sup>P]ATP (3000 Ci/mmol, Perkin Elmer) and 3  $\mu$ M tRNA nucleotidyltransferase. After incubation at 37°C for 5 min, yeast pyrophosphatase (PPase, Roche) was added to a final concentration of 10 U/ml, and the reaction mixture was incubated for an additional 2 min before quenching by phenol extraction.

Incorporation of unlabeled Leu, Nva or ABA amino acids onto 3'-<sup>32</sup>P-labeled tRNA<sup>Leu</sup> was measured by aminoacylation assays which were identical to those described above except that 5 mM Leu, 10 mM Nva or 15 mM ABA was used. After aminoacylation, all reactions were stopped and products digested by placing 1  $\mu$ l of reaction mixture in a buffer containing 200  $\mu$ M sodium acetate (pH 5.0) and 0.2 U/ $\mu$ l P1 nuclease (Fluka) and incubating for 10 min at 25°C. 3'-aminoacylated A76 from aminoacylated tRNA and AMP from uncharged tRNA were separated by TLC and quantified by phosphorimaging analysis as described above (41).

## RESULTS

### Overexpression of hcLeuRS and hctRNA<sup>Leu</sup> in *E. coli* improves aminoacylation

In our earlier study, the gene encoding hcLeuRS was expressed in insect cells (33). Here, we cloned the human gene into the plasmid pET22b in order to overexpress the protein in *E. coli* with an Ala<sub>3</sub>-His<sub>6</sub> tag at the C-terminus. From 1.5 g of wet cells, about 2 mg of hcLeuRS were obtained. The enzyme was purified to 90% homogeneity with a molecular mass of 135 kDa, which was similar to the one purified from the baculovirus system (33). HcLeuRS overexpressed in *E. coli* exhibited the same *k*<sub>cat</sub> values as the one overexpressed in insect cells and reached around 0.3 s<sup>-1</sup> with the transcript of hctRNA<sup>Leu</sup> (Table 1).

In an attempt to improve the *k*<sub>cat</sub> value of the aminoacylation reaction, hctRNA<sup>Leu</sup> was cloned in the bacterial expression vector pTrec99B (36,37) in order to produce a tRNA containing the base modifications that are often essential for tRNA structure and stability. After *E. coli* transformation and IPTG induction, 34 mg of crude tRNA were isolated from 6 g of wet cells by the standard phenol extraction procedure. This tRNA exhibited a leucine-accepting activity of 864 pmol/A<sub>260</sub> with hcLeuRS but only 103 pmol/A<sub>260</sub> with *E. coli* LeuRS (*EcLeuRS*) (data not shown). After fractionation of the total tRNA by DEAE-Sepharose chromatography, the leucylation activity of the tRNA was improved to



**Table 1.** Kinetic constants of hcLeuRS for various tRNA<sup>Leu</sup>s in the aminoacylation reaction

Constants	Calf liver tRNA <sup>Leu</sup> (50 pmol/A <sub>260</sub> ) <sup>a</sup>	<i>In vitro</i> transcribed hctRNA <sup>Leu</sup> (680 pmol/A <sub>260</sub> )	Overexpressed <i>Ect</i> RNA <sup>Leu</sup> (1400 pmol/A <sub>260</sub> )	Overexpressed hctRNA <sup>Leu</sup> (1600 pmol/A <sub>260</sub> )
$K_M$ (μM)	1.9 ± 0.2	1.02 ± 0.04	0.58 ± 0.02	0.74 ± 0.05
$k_{cat}$ (s <sup>-1</sup> )	0.28 ± 0.02	0.33 ± 0.01	0.45 ± 0.04	2.56 ± 0.20
$k_{cat}/K_M$ (s <sup>-1</sup> mM)	147.4	323.5	775.9	3459.5

<sup>a</sup>Values from Ling *et al.* (2005).

All data in this table are the average values from three independent determinations.

**Table 2.** Kinetic constants of hcLeuRS obtained from *E. coli* and baculovirus in the aminoacylation reaction

Substrates	Constants	hcLeuRS (from <i>E. coli</i> )	hcLeuRS <sup>a</sup> (from baculovirus)
Leucine	$K_M$ (μM)	2.97 ± 0.07	7.5 ± 0.2
	$k_{cat}$ (s <sup>-1</sup> )	2.69 ± 0.03	0.30 ± 0.04
	$k_{cat}/K_M$ (s <sup>-1</sup> mM)	905.7	40
ATP	$K_M$ (μM)	112.1 ± 2.1	773 ± 70
	$k_{cat}$ (s <sup>-1</sup> )	2.37 ± 0.03	0.27 ± 0.01
	$k_{cat}/K_M$ (s <sup>-1</sup> mM)	21.14	0.35

<sup>a</sup>Values from Ling *et al.* (2005).

All data in this table are the average values from three independent determinations.

1450 pmol/A<sub>260</sub>, and then to 1600 pmol/A<sub>260</sub> via further fractionation on a C4 reversed-phase HPLC column. Finally, about 4.5 mg of nearly 100% pure hctRNA<sup>Leu</sup> were obtained starting from 6 g of wet cells. The isolated hctRNA<sup>Leu</sup> had more thermal stability as the melting point of the hctRNA<sup>Leu</sup> was 15°C higher than that of its transcript (data not shown).

In the aminoacylation reaction catalyzed by hcLeuRS, the recombinant hctRNA<sup>Leu</sup> that was overexpressed and purified in *E. coli* was the most efficient substrate. The  $k_{cat}$  value (2.56 s<sup>-1</sup>) for hctRNA<sup>Leu</sup> isolated from *E. coli* was 9.1-, 7.8- and 5.7-fold higher compared to that of crude calf liver tRNA (0.28 s<sup>-1</sup>), hctRNA<sup>Leu</sup> transcript (0.33 s<sup>-1</sup>), and *Ect*RNA<sup>Leu</sup> (0.45 s<sup>-1</sup>), respectively. The  $K_M$  value was the lowest among the four kinds of tRNA<sup>Leu</sup>s and the catalytic efficiency of the present hcLeuRS for hctRNA<sup>Leu</sup> was 23-, 10- and 4-fold higher compared to those above tRNA<sup>Leu</sup>s, respectively (Table 1). Thus, the expression of both human LeuRS and tRNA<sup>Leu</sup> in *E. coli* led to a system with an efficiency that is comparable to that of the *Ec*LeuRS for *Ect*RNA<sup>Leu</sup> (14). Overall, these results showed that the overexpressed hctRNA<sup>Leu</sup> is a competent substrate of hcLeuRS for the aminoacylation and editing study.

We also analyzed the Michaelis constants of hcLeuRS overexpressed in *E. coli* for Leu and ATP in the aminoacylation reaction and compared them with those from the enzyme expressed in baculovirus (33). Although the  $k_{cat}$  values were nearly the same, the  $K_M$  values for Leu and ATP were both reduced, showing that the enzyme binds more tightly to these substrates. This resulted in an increase of the catalytic efficiency ( $k_{cat}/K_M$ ) in the

aminoacylation reaction of 60.4-fold for ATP and 22.6-fold for Leucine (Table 2), confirming that the enzyme overexpressed in *E. coli* has a robust activity and is ideal for subsequent studies.

### HcLeuRS misactivates several non-cognate amino acids

It has been shown previously that Ile and Met as well as the non-standard amino acids norvaline (Nva) and α-amino butyrate (ABA) are misactivated by LeuRS (5,14,42,43). Among them, ABA is the smallest molecule and Nva is the analog most similar to Leu. The kinetic parameters of hcLeuRS for these various amino acids were determined in the activation reaction; however, the Mg<sup>2+</sup>/ATP ratio was optimized and increased from 0.5 (33) to 3 prior to performing the assays. Consequently, this resulted in an increase of the  $k_{cat}$  from 0.8 to 25.8 s<sup>-1</sup>, a value much higher than the recently published value of 2.1 s<sup>-1</sup> (measured with an Mg<sup>2+</sup>/ATP ratio of 10) (34). The non-cognate amino acid activation assays showed that Ile and Met were poorly activated by the human enzyme (Table 3). In the present work, the misactivation frequency for Ile was lower than the mis-translation frequency (1/3300) in protein biosynthesis (44), suggesting hcLeuRS would not require an editing mechanism to prevent mischarging of this amino acid. On the other hand, Met was more efficiently misactivated by hcLeuRS, indicating that the amino acid should be edited *in vivo* to prevent mis-incorporation into nascent proteins (Table 3).

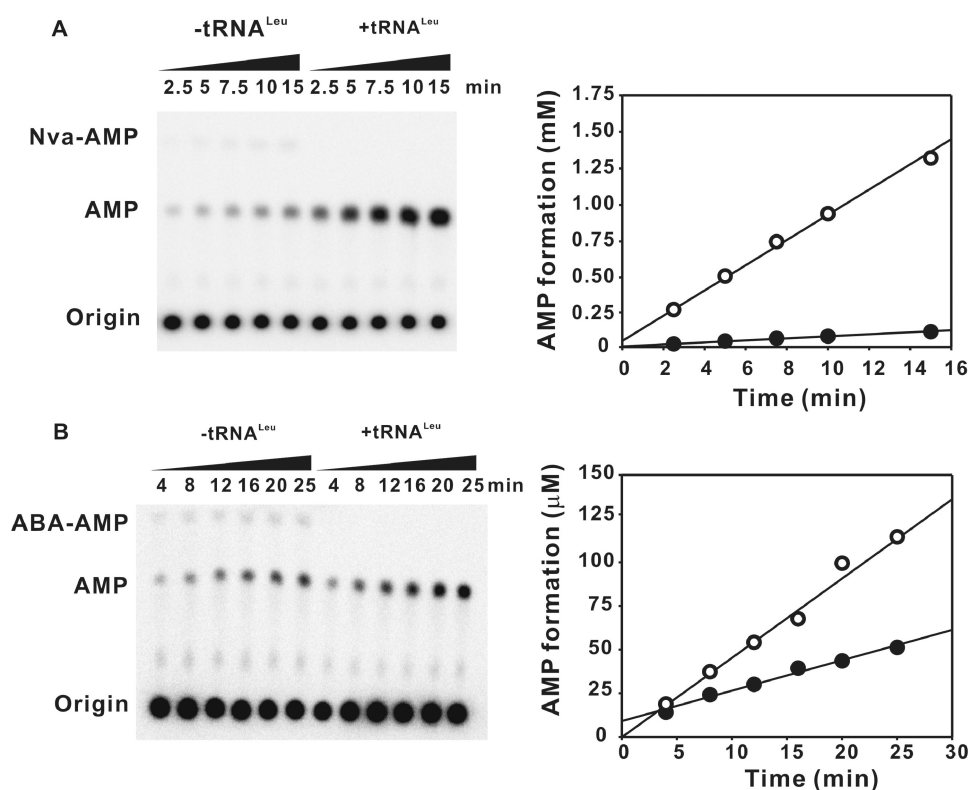
Among the non-standard amino acids, Nva was the most efficiently misactivated compound. HcLeuRS to Nva exhibited discrimination factors of about 100 compared to Leu. The  $k_{cat}$  for Nva activation decreased only about half of that for Leu, and the main effect was a 48-fold increase of  $K_M$  as compared with for Leu. The  $k_{cat}$  value for ABA misactivation was much higher than those for Ile and Met, and was only 6.6-fold lower than that of Leu. However, the catalytic efficiency was quite low because of the very high  $K_M$  value of hcLeuRS for ABA, which was about 800-fold of that for Leu (Table 3). Altogether, these data showed that the synthetic site of hcLeuRS could accommodate various non-cognate amino acids. This also suggests that the enzyme might have evolved proofreading pathways to get rid of misactivated compounds to prevent genetic code ambiguity.

**Table 3.** Kinetic constants of wild-type and D399A mutant of hcLeuRS in amino acid activation reaction

hcLeuRS	Amino Acids	$K_M$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_M$ ( $\text{s}^{-1} \text{mM}^{-1}$ )	Discrimination factor <sup>a</sup>
WT	Leu	45.6 $\pm$ 9.5	25.8 $\pm$ 1.5	565	1
	Ile	1248 $\pm$ 254	0.162 $\pm$ 0.028	0.13	4346
	Met	1547 $\pm$ 334	0.474 $\pm$ 0.130	0.31	1823
	Nva	2195 $\pm$ 68	12.4 $\pm$ 0.5	5.65	100
	ABA	36 200 $\pm$ 2600	3.91 $\pm$ 0.15	0.108	5231
D399A	Leu	50.0 $\pm$ 8.8	26.2 $\pm$ 0.9	524	1
	Nva	5135 $\pm$ 553	13.7 $\pm$ 1.9	2.67	196
	ABA	49 440 $\pm$ 7350	4.1 $\pm$ 0.1	0.083	6313

<sup>a</sup>Corresponds to the loss of catalytic efficiency relative to Leu.

The data are the average values from three independent experiments.



**Figure 1.** Formation of [<sup>32</sup>P]AMP and AA-[<sup>32</sup>P]AMP catalyzed by hcLeuRS. Reactions were performed with (A) Nva or (B) ABA, in the absence (-tRNA<sup>Leu</sup>) or presence (+tRNA<sup>Leu</sup>) of tRNA<sup>Leu</sup>. The graphs represent the quantification of the AMP formation in the presence of tRNA<sup>Leu</sup> (open circle) and in its absence (closed circle).

### Nva is mainly edited by tRNA-dependent mechanisms

Each time that a non-cognate amino acid is activated by LeuRS and then hydrolyzed, one molecule of ATP is transformed into AMP. Successive rounds of activation/editing lead to accumulation of AMP molecules that can be tracked by thin layer chromatography (TLC). TLC can also be used to monitor the aminoacyl-adenylate formation (41). To perform the editing assay, enzymes were incubated with  $\alpha$ -[<sup>32</sup>P]ATP, non-cognate amino acid and tRNA<sup>Leu</sup>. Aliquots of the editing mixtures were quenched and applied on PEI TLC plates and the [<sup>32</sup>P]AMP formation rates for hcLeuRS and mutant derivatives were calculated (Figure 1, Table 4). In the assay, cognate Leu induced a very low level of AMP, which is in agreement

with the fact that Leu is activated but not edited by LeuRS (Supplementary Figure S1).

Editing of Nva was first assayed because of its relatively high misactivation rate compared with Ile and Met. In the absence of tRNA, the AMP formation rate with Nva reached 0.119 s<sup>-1</sup>. In theory, this AMP formation corresponded mainly to the tRNA-independent pre-transfer editing activity, which was added to the very low level of spontaneous hydrolysis of the released adenylate molecules (1.63  $\times$  10<sup>-4</sup> s<sup>-1</sup>, Supplementary Figure S2). The presence of tRNA<sup>Leu</sup> in the activation reaction mix stimulated the AMP formation by about 13-fold (1.57 s<sup>-1</sup>). This showed that hcLeuRS carried a strong tRNA-dependent editing activity that represented more

**Table 4.** Observed steady-state constants of hcLeuRS and its D399A mutant in AMP and aa-AMP synthesis reaction

hcLeuRS	Amino acids	tRNA <sup>Leu</sup>	AN2690	AMP formation $k_{\text{obs}}$ (s <sup>-1</sup> )	AA-AMP formation $k_{\text{obs}}$ (s <sup>-1</sup> )
WT	Nva	—	—	$(1.19 \pm 0.12) \times 10^{-1}$	$(1.21 \pm 0.12) \times 10^{-2}$
	Nva	+	—	$1.57 \pm 0.08$	nd
	Nva	—	+	$(1.16 \pm 0.09) \times 10^{-1}$	$(1.29 \pm 0.19) \times 10^{-2}$
	Nva	+	+	$(2.52 \pm 0.31) \times 10^{-1}$	$(0.46 \pm 0.08) \times 10^{-2}$
	Leu	—	—	$(5.27 \pm 0.68) \times 10^{-3}$	nd
	Leu	+	—	$(9.85 \pm 1.31) \times 10^{-3}$	nd
	ABA	—	—	$(3.19 \pm 0.28) \times 10^{-2}$	$(3.27 \pm 0.41) \times 10^{-3}$
	ABA	+	—	$(8.69 \pm 1.11) \times 10^{-2}$	nd
	ABA	—	+	$(3.47 \pm 0.24) \times 10^{-2}$	$(3.77 \pm 0.83) \times 10^{-3}$
	ABA	+	+	$(7.82 \pm 1.72) \times 10^{-2}$	nd
D399A	Nva	—	—	$(9.91 \pm 2.10) \times 10^{-2}$	$(0.71 \pm 0.13) \times 10^{-2}$
	Nva	+	—	$(1.03 \pm 0.25) \times 10^{-1}$	$(0.72 \pm 0.09) \times 10^{-2}$
	ABA	—	—	$(1.31 \pm 0.33) \times 10^{-2}$	$(5.46 \pm 1.09) \times 10^{-4}$
	ABA	+	—	$(3.11 \pm 0.87) \times 10^{-2}$	$(2.09 \pm 0.28) \times 10^{-3}$

Data in this table are the average values from three independent determinations.

nd: not detected.

than 92% of the total editing activity. The result was consistent with the values obtained with *Aquifex aeolicus* LeuRS and Nva under the same conditions (28,45).

The TLC plates also revealed the accumulation of Nva-AMP in the absence of tRNA<sup>Leu</sup> when the enzyme only exhibited tRNA-independent pre-transfer editing (Figure 1A, Table 4). The concentration of Nva-AMP exceeded the enzyme concentration, suggesting that it resulted from multiple turnovers and release in solution. However, when tRNA<sup>Leu</sup> was present, Nva-AMP no longer accumulated, indicating that it was more efficiently edited after its transfer onto the tRNA, or alternatively by tRNA-dependent pre-transfer editing.

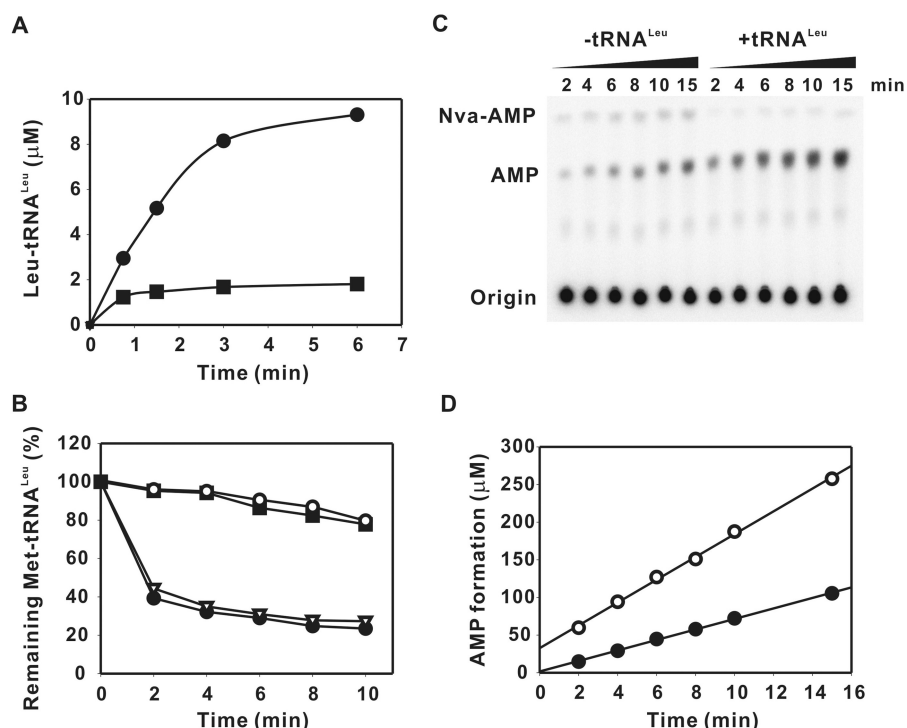
### Separation of the Nva-editing pathways

To investigate more thoroughly the editing pathways of Nva and to separate the AMP formation resulting from post-transfer editing and tRNA-dependent pre-transfer editing, two additional assays were performed.

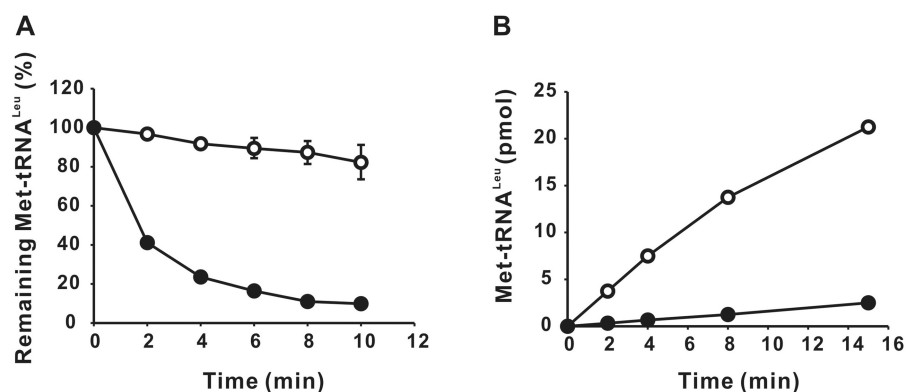
First, AMP formation assays were performed in the presence of the recently identified benzoxaborole antifungal compound AN2690. This molecule targets LeuRS specifically by forming a covalent adduct with the 3'-adenosine of tRNA<sup>Leu</sup> at the post-transfer editing site (46,47). In that complex, tRNA is locked in an inactive conformation for aminoacylation and post-transfer editing (46,47) while both tRNA-dependent or -independent pre-transfer editing pathways are kept intact. In the present work, AN2690 was tested against human LeuRS and was shown to inhibit Leu-tRNA<sup>Leu</sup> formation as previously observed for fungal LeuRSs (46) and *E. coli* LeuRS (19). With 100  $\mu$ M of AN2690, the aminoacylation activity of hcLeuRS was considerably reduced and the charging plateau did not reach 20% (Figure 2A). Similarly, the hydrolytic capacity of mischarged Met-tRNA<sup>Leu</sup> was drastically reduced in the presence of AN2690 (Figure 2B). These effects were consistent with the previous results and confirmed that locking the tRNA acceptor end into the editing site was

deleterious for both aminoacylation and post-transfer editing activities. In contrast, when AMP formation was measured in editing conditions in the presence of Nva and AN2690, a stimulation of AMP formation was observed by the tRNA ( $0.252 \text{ s}^{-1}$ ) compared to the reaction without tRNA ( $0.116 \text{ s}^{-1}$ ) (Table 4, Figure 2C and D). This suggests that a tRNA-dependent editing pathway was still active in the presence of AN2690, and this pathway should be a pre-transfer pathway since the acceptor end of the tRNA was locked in an inactive conformation. This also showed that tRNA binding in the editing site is required for tRNA-dependent pre-transfer editing, a conclusion already reached with a study previously performed on *E. coli* LeuRS (19).

Second, a CP1 mutation was used to abolish the post-transfer editing pathway. Residue D399 in hcLeuRS is conserved amino acid residue, which is homologous to D345 in *E. coli* (48), D419 in yeast cytoplasmic LeuRS (ycLeuRS) (49), and D373 in *A. aeolicus* LeuRS (AaLeuRS) (28). In *Thermus thermophilus* LeuRS, this Asp residue forms salt bridge interactions with the amino group of the Nva moiety in both the pre- and post-transfer analogs (48). In LeuRSs, the mutation of this invariant Asp residue inactivated the post-transfer editing (28,34,49). We mutated the conserved D399 to A of hcLeuRS to obtain hcLeuRS-D399A and measured the amino acid activation, tRNA charging, and editing activities of the D399A mutant. HcLeuRS-D399A had full activity in the amino acid activation of Leu (Table 3). Post-transfer editing activity of hcLeuRS-D399A was also determined by the deacylation of [<sup>3</sup>H]-Met-tRNA<sup>Leu</sup>. In 2 min, wild-type hcLeuRS (25 nM) deacylated 60% of the Met-tRNA<sup>Leu</sup>; however, the D399A failed to hydrolyze Met-tRNA<sup>Leu</sup> under the same conditions (Figure 3A) (28,34,49). This obvious loss of post-transfer editing activity of hcLeuRS-D399A was accompanied by an increase of the misacylation activity compared with the wild-type hcLeuRS (Figure 3B). Taken together, these results indicated that the conserved Asp residue is absolutely crucial for



**Figure 2.** Effect of AN2690 on the aminoacylation and editing properties of hcLeuRS. (A) Aminoacylation of 10 μM tRNA<sup>Leu</sup> by 25 nM hcLeuRS in the presence (closed square) or absence (closed circle) of AN2690 (100 μM). (B) Hydrolysis of 1 μM Met-tRNA<sup>Leu</sup> by 25 nM hcLeuRS (closed circle), in the presence of 5 μM tRNA<sup>Leu</sup> (open triangle), or in the presence of 5 μM tRNA<sup>Leu</sup> and 100 μM AN2690 (closed square). Spontaneous hydrolysis of Met-tRNA<sup>Leu</sup> is also presented in the presence of 100 μM AN2690 (open circle). (C) AMP formation in the presence or absence of tRNA (5 μM tRNA<sup>Leu</sup>) with 100 μM AN2690 and 10 mM Nva. (D) Graphical representation of the TLC data shown in (C), in the presence (open circle) or absence (closed circle) of tRNA<sup>Leu</sup>.

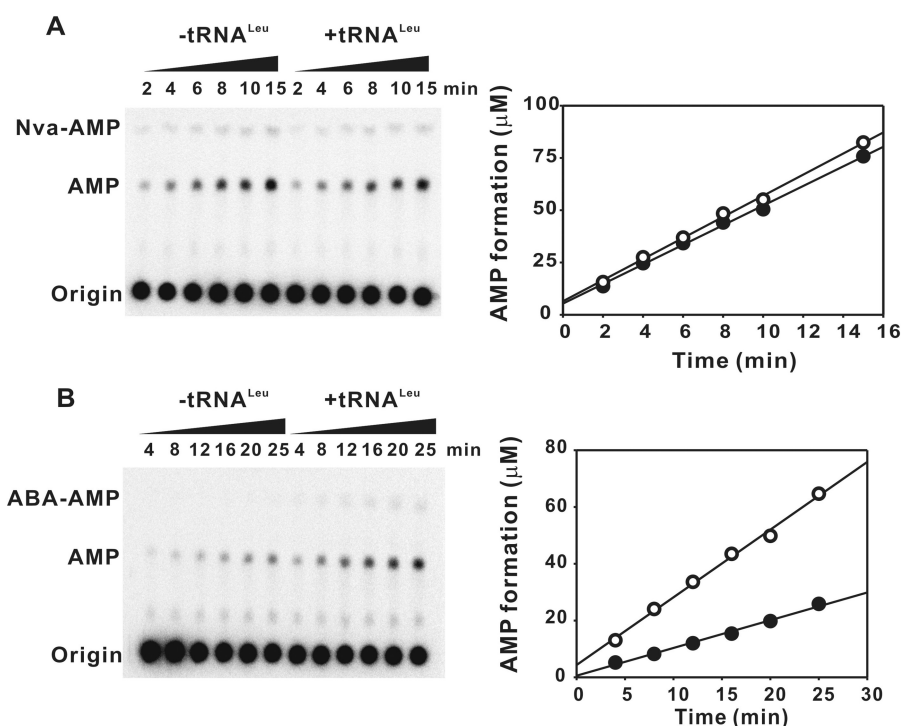


**Figure 3.** Effects of the D399A mutation on the editing properties of hcLeuRS. (A) Hydrolysis of Met-tRNA<sup>Leu</sup> by 25 nM hcLeuRS (closed circle) and hcLeuRS-D399A (open circle). (B) Production of Met-tRNA<sup>Leu</sup> by 1.5 μM hcLeuRS (closed circle) and D399A mutant (open circle).

post-transfer editing of hcLeuRS and for enzyme fidelity since a significant amount of mis-charged tRNA is formed by its mutation. This is consistent with previously reported studies with *Aa*LeuRS, *hc*LeuRS, *Ec*LeuRS and *yc*LeuRS (28,34,48,49). Furthermore, the AMP formation catalyzed by wild-type LeuRS and the mutant hcLeuRS-D399A were assayed in editing conditions. The AMP formation rate catalyzed by hcLeuRS-D399A was almost the same as that of the wild-type enzyme ( $k_{\text{obs}}$  0.0991 s<sup>-1</sup> versus 0.119 s<sup>-1</sup>) with Nva and in the absence of tRNA

(Figure 4A, Table 4). Therefore, the CP1-mutation did not change the tRNA-independent editing process. In the presence of tRNA and Nva, AMP formation by hcLeuRS-D399A was only increased slightly as compared with that without tRNA ( $k_{\text{obs}}$  0.103 s<sup>-1</sup> and 0.0991 s<sup>-1</sup>, respectively), indicating that tRNA-dependent editing activity is affected by the mutation of CP1 domain, because the  $k_{\text{obs}}$  of AMP formation with tRNA by wild type hcLeuRS was increased 13-fold than that without tRNA.





**Figure 4.** Formation of [ $^{32}$ P]AMP and AA-[ $^{32}$ P]AMP catalyzed by hcLeuRS-D399A. [ $^{32}$ P]ATP, [ $^{32}$ P]AMP, AA-[ $^{32}$ P]AMP were separated by TLC. Reactions were carried out with (A) Nva and (B) ABA by hcLeuRS-D399A, in the absence (-tRNA<sup>Leu</sup>) or presence (+tRNA<sup>Leu</sup>) of tRNA<sup>Leu</sup>. The graphs represent the quantification of the AMP formation in the presence of tRNA<sup>Leu</sup> (open circle) and in its absence (closed circle).

The results show that pre-transfer pathways account for about 15% of total editing and is nearly equally split between tRNA-dependent (9% of total editing) and tRNA-independent (7%) mechanisms. The post-transfer pathway is far more significant, accounting for 84% of the total editing (Figure 5A).

#### ABA is mainly edited by pre-transfer editing pathways

To examine if hcLeuRS edits other non-cognate amino acids using the same editing pathways, the former assays were repeated in the presence of ABA. ABA generates toxicity in bacterial cells containing ValRS or LeuRS deprived of editing activities (42,50). This observation strongly suggested that ABA was misactivated *in vivo* and then transferred onto the corresponding tRNAs before entering protein synthesis. We assayed the editing reaction of hcLeuRS in the presence of ABA (Figure 1B); however, the catalytic efficiency of the activation reaction measured in the ATP-PPi exchange reaction was very low as compared to Nva, Ile and Met *in vitro* (Table 3). This was mainly due to a severe increase of the  $K_M$  for ABA that reached 36 mM. To bypass this problem, the concentration of ABA was increased to 15 mM in the editing assay. Under these conditions, the AMP formation rate reached  $0.0319\text{ s}^{-1}$  and  $0.0869\text{ s}^{-1}$  in the absence and the presence of tRNA, respectively (Table 4). Editing of ABA disproportionally produced more AMP by the tRNA-independent pathway, with 37% ( $3.19/8.69$ ) of the total editing pathway, compare to Nva editing (7%). This result suggests hcLeuRS could modulate its

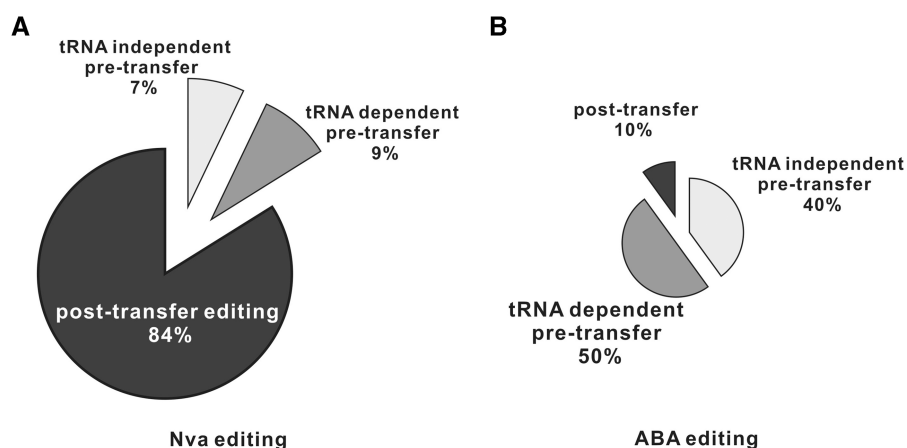
preference of the editing pathways according to the identity of the amino acid.

The editing assay was also measured in the presence of AN2690, which inhibits tRNA post-transfer editing. The AMP formation rates were  $0.0347\text{ s}^{-1}$  and  $0.0782\text{ s}^{-1}$  in the absence and presence of tRNA, respectively (Table 4, Supplementary Figure S1). According to these data, tRNA-dependent pre-transfer editing pathway contributed 50% [ $(7.82 - 3.47)/8.69$ ] to total editing activity, and post-transfer editing pathway only 10% [ $(8.69 - 7.82)/8.69$ ], showing that hcLeuRS prefers the pre-transfer editing pathway to edit ABA (Figure 5B).

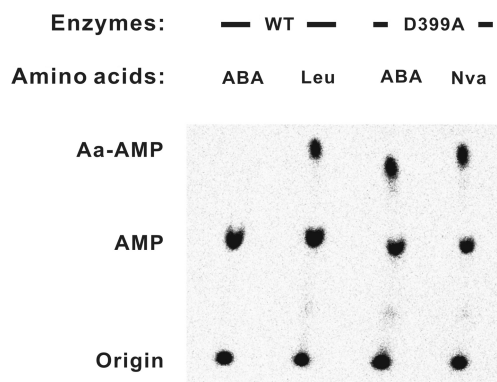
AMP formation in the presence of ABA was also assayed with the hcLeuRS-D399A mutant (Figure 4B). In the absence of tRNA, the editing activities were decreased compared to the wild-type hcLeuRS ( $0.0131\text{ s}^{-1}$  and  $0.0319\text{ s}^{-1}$ , respectively). This decrease of AMP formation rates by the D399A mutant may be explained partially by the slight increase of  $K_M$  for ABA in the activation rate (Table 3). In the presence of tRNA,  $k_{\text{obs}}$  of the AMP formation to edit ABA by hcLeuRS-D399A increased to  $0.0311\text{ s}^{-1}$ . The D399A mutation destroys post-transfer editing. Because it has minor effects on tRNA-dependent editing, pre-transfer editing is more significant for ABA.

A significant amount of ABA-AMP was formed in the absence of tRNA with a rate reaching one-tenth of the AMP formation rate (Figure 1B). In the assay, the ABA-AMP concentration exceeded the enzyme concentration, which means that it was released in the solution, where it was found to be hydrolyzed spontaneously at a





**Figure 5.** Summary of the relative contributions of the different editing pathways used by hcLeuRS to edit Nva and ABA. HcLeuRS edits (A) Nva predominantly by post-transfer editing while uses mainly pre-transfer editing to edit (B) ABA. The global editing activity was measured as AMP formation in the presence of tRNA. The contribution of the tRNA-independent pre-transfer editing was measured in the absence of tRNA. The contribution of the tRNA-dependent pre-transfer editing was deduced from the AMP formation measured in the presence of the AN2690 compound. The post-transfer editing accounted for the left in the global editing.



**Figure 6.** Aminoacylation of  $\text{hcLeuRS}^{\text{Leu}}$  with Leu, Nva and ABA performed by the hcLeuRS or hcLeuRS-D399A. Aminoacylations were carried out with  $5\mu\text{M}$   $^{32}\text{P}$ -labeled  $\text{hcLeuRS}^{\text{Leu}}$ ,  $5\text{mM}$  Leu or  $10\text{mM}$  Nva or  $15\text{mM}$  ABA by  $1.5\mu\text{M}$  hcLeuRS-D399A or  $500\text{nM}$  hcLeuRS, respectively. AMP (from uncharged tRNA) and aminoacyl-AMP (from aminoacyl-tRNA) after nuclease P1 digestion of aminoacylation reactions were separated by TLC.

rate of  $4.63 \times 10^{-4} \text{ s}^{-1}$  (Supplementary Figure S2). This is considerably lower than the tRNA-independent AMP formation rate ( $0.0319 \text{ s}^{-1}$ ). Likewise, when  $\text{tRNA}^{\text{Leu}}$  was added to the editing solution, the non-cognate adenylate did not accumulate, suggesting it was edited through a process that was tRNA-dependent (Table 4).

#### Nva-/ABA-tRNA<sup>Leu</sup> can be formed by the hcLeuRS-D399A mutant

Nva and ABA are not commercially available as radio-labeled compounds, and therefore  $\text{tRNA}^{\text{Leu}}$  charging with Nva and ABA cannot be measured by routine methods. We used a highly sensitive assay to directly determine formation of Nva-tRNA<sup>Leu</sup> and ABA-tRNA<sup>Leu</sup>, which is based on the internal  $^{32}\text{P}$  labeling of the last phosphate linkage of tRNA (40,41). After aminoacylation of the 3'-end  $^{32}\text{P}$ -labeled tRNA, the tRNA was hydrolyzed by the nuclease P1, and the mixture containing labeled aminoacyl-AMP from charged tRNA

and labeled AMP from uncharged tRNA were separated by TLC.

HcLeuRS-D399A could catalyze the aminoacylation of  $\text{tRNA}^{\text{Leu}}$  with Nva or ABA to form Nva-tRNA or ABA-tRNA, which were clearly observed on the thin plate (Figure 6). On the other hand, wild-type hcLeuRS could only form  $\text{Leu-tRNA}^{\text{Leu}}$  based on the fact that the Leu-AMP band but not the ABA-AMP band was seen on the TLC plate. Furthermore, the absence of ABA-AMP combined with the data from Table 4 indicated that native LeuRS could hydrolyze effectively with either ABA-tRNA or misactivated ABA. Taken together, these data suggest non-cognate amino acids such as Nva and ABA could be charged onto  $\text{tRNA}^{\text{Leu}}$  when the post-transfer editing function of hcLeuRS is destroyed, which is consistent with the previous *in vivo* results in *E. coli* (43,50,51). For ABA editing, although the pre-transfer pathway is dominant, the post-transfer pathway is still necessary to prevent the mischarge by hcLeuRS.

## DISCUSSION

### The hcLeuRS and $\text{hcLeuRS}^{\text{Leu}}$ expressed in *E. coli* provide an efficient system for studying hcLeuRS function

In mammals, cytoplasmic LeuRS is one of the eight components of the MSC. The properties of hcLeuRS have only been partially studied previously. Our lab had reported on the expression and purification of recombinant hcLeuRS from insect cells (33). This hcLeuRS was shown to have high specific activity and its C-terminal extension was crucial for the interaction with the N-terminal extension of hcArgRS (33). In order to further evaluate the editing properties of hcLeuRS, we simplified its expression and purification method. The present preparation was obtained from *E. coli* transformants and purified by affinity chromatography on a  $\text{Ni}^{2+}$ -NTA column. As compared with the previous preparation from the baculovirus system, its activities of

amino acid activation and aminoacylation of tRNA<sup>Leu</sup> transcript were considerably improved by the rapid protocol and the optimal Mg<sup>2+</sup>/ATP ratio. HcLeuRS had exceptionally high charging catalytic efficiency with hctRNA<sup>Leu</sup> purified from *E. coli* overproducing strain, and was 8-fold higher than that of the tRNA<sup>Leu</sup> transcript (Table 1). Transfer RNAs are involved in translation of the genetic code as well as gene expression regulation (52,53). It has been known for a long time that modified bases of tRNA are very important for tRNA structure and function (54–57). Modified bases may constrain the tRNA structure (55) and provide interacting groups with the enzyme (58–60). Human cytoplasmic tRNA<sup>Leu</sup> isolated from *E. coli* transformants exhibited higher thermal stability, higher accepting capacity, and although we did not study tRNA structure directly, this could be due to the modification of some nucleotides during expression. Thus, our present hctRNA<sup>Leu</sup> isolated from *E. coli* transformants provide an efficient system to study the function and tertiary structure of hctRNA<sup>Leu</sup>, which will be helpful in designing novel selective antibiotics targeted to the synthetic and editing active sites of LeuRSs from pathogenic bacteria.

### HcLeuRS misactivates Nva and ABA

LeuRS is closely related to IleRS and ValRS, belonging to the LIV-RS subgroup of class Ia synthetases (1). Leu, Ile and Val are hydrophobic amino acids that differ slightly. Incidentally, when misactivation errors occur, IleRS must eliminate the non-cognate amino acid valine (10). ValRS edits the non-cognate, isosteric threonine, which only differs by a hydroxyl group (12) and LeuRS has to discriminate leucine from isoleucine and methionine that are mischarged on tRNA<sup>Leu</sup> (14). In addition to these amino acids, various non-protein amino acids are activated by LeuRS (13). Here, the activation properties of hctRNA<sup>Leu</sup> for two non-protein amino acids were studied. First, Nva was chosen since this compound is naturally found *in vivo* (61,62), and can be incorporated into proteins (51). Compared to Leu, Nva differs by the absence of a single methyl group and consequently was misactivated by hctRNA<sup>Leu</sup> with a loss of catalytic efficiency of only 100-fold compared with Leu (Table 3). This loss was mainly due to a  $K_M$  effect corresponding to a binding energy variation of only 2.7 kcal/mol, which is consistent with the loss of van der Waals forces expected from interaction of a methyl group with a hydrophobic binding site. HcLeuRS and *E. coli* or *A. aeolicus* LeuRSs exhibited nearly the same binding energy variation (19,28), suggesting that Nva binds and reacts with the three enzymes in a similar way.

In parallel, ABA misactivation by LeuRS was analyzed. ABA has been shown to generate toxicity in *E. coli* cells exhibiting ValRS and LeuRS deprived of editing activities (42,50), which suggests ABA was misactivated before being charged on tRNA<sup>Leu</sup> and subsequently incorporated into proteins. Therefore, the activation of ABA by hctRNA<sup>Leu</sup> was investigated. As ABA is smaller than Leu and Nva, it was predicted that there should be no steric hindrance to the active site of LeuRS. Indeed, the  $k_{cat}$  of

hctRNA<sup>Leu</sup> for ABA in activation reaction was reduced, but remained in the same order of magnitude as the  $k_{cat}$  for Leu. On the other hand, the  $K_M$  for ABA was increased by 800-fold for Leu, which indicated that part of the binding energy was lost during binding of the smaller molecule (5 kcal/mol). Altogether, these results suggest Nva and ABA could potentially be activated by hctRNA<sup>Leu</sup> *in vivo*, especially when concentrations of these compounds are high, and therefore may require editing mechanisms to clear the misactivated or mischarged products.

### Nva is preferentially edited by post-transfer editing pathway

The present study showed that hctRNA<sup>Leu</sup> used several editing pathways to exclude the wrong products. The tRNA-dependent editing pathways formed the highest amounts of AMP (93%), whereas the tRNA-independent editing pathways only accounted for 7% of the AMP formation calculated from the kinetic parameters in Table 4. Basically, the tRNA-dependent editing pathways include tRNA-dependent pre-transfer and post-transfer editing (19). The tRNA-independent editing pathways group tRNA-independent pre-transfer pathway and spontaneous hydrolysis of non-cognate after release in solution. Thanks to the recently developed antifungal drug AN2690, it was possible to inhibit specifically the post-transfer editing reaction and isolate the tRNA-dependent pre-transfer editing reaction (46,47). In this way, the tRNA-dependent pre-transfer editing reaction was found to account for 9% of the total AMP formation, a value close to the tRNA-independent pre-transfer editing that was measured in the absence of tRNA (7%). These two reactions were rather low compared to the post-transfer editing, which contributed to about 84% of the AMP formation to edit Nva. The last pathway, non-enzymatic hydrolysis or ‘kinetic proofreading’ (30,63), was measured by inducing a chase with ATP. According to previous studies of LeuRSs (19,28), this pathway is also a minor editing pathway for hctRNA<sup>Leu</sup> (Supplementary Figure S2) and contributed to <1% of the total AMP formation rate. It is likely that the partition of the editing activities in three main pathways is not static but follows changes and adaptations to the physiological conditions. Herein, the quantification of each pathway was obtained by shutting down the other pathways by different methods (D399A mutation in the editing site, AN2690 compound and the absence of tRNA). It is possible that in response to the suppression of one editing pathway the other might have been activated as suggested earlier (64). Overall, these percentages should not be regarded as definitive values, but they highlight the enzyme resources to get rid of incorrect intermediates or products.

### ABA is mainly edited by pre-transfer editing mechanisms

The AMP formation assays performed in the presence of ABA were only weakly stimulated by tRNA<sup>Leu</sup> compared to similar assays performed with Nva. In the presence of tRNA and AN2690, which targeted the post-transfer editing reaction, the AMP formation rate was slightly

decreased when compared with the reaction without AN2690 (0.0782 versus 0.0869 s<sup>-1</sup>). This suggests that when post-transfer editing pathway of hcLeuRS is blocked by AN2690, tRNA-dependent pre-transfer editing pathway is the exclusive contributor to ABA editing. Moreover, we can estimate that the post-transfer pathway accounts for only 10% of the editing. Here, we showed that ABA can be activated by hcLeuRS to form ABA-AMP (Figure 1B). In addition, we used a sensitive assay to determine the mis-charged products (Nva- or ABA-tRNA<sup>Leu</sup>) based on the labeling of the 3'-last phosphate of tRNA<sup>Leu</sup> by tRNA nucleotidyltransferase. The data showed that Nva and ABA can be charged on the tRNA by the editing-defective but not native enzyme (Figure 6), because hcLeuRS-D399A decreased the activity to edit Nva and ABA. Although post-transfer editing activity to edit ABA represents only 10% of total editing activity of the native hcLeuRS, this activity is high enough to deacylate ABA. In contrast, hcLeuRS-D399A is deprived of post-transfer editing and accumulates ABA-tRNA. The results suggested that post-transfer editing is the last but crucial checkpoint for maintaining the fidelity of aminoacylation of aaRSs, even if it only contributes to a minor part of the total editing activity. For this reason, the post-transfer editing is essential and a slight decrease of this editing activity may affect the aminoacylated products synthesized by aaRSs. It has been shown that cellular accumulation of mis-charged products induces mistranslation and then cell death, causing diseases such as neurodegeneration (65,66).

#### Adaptation of enzyme editing properties to distinct misactivated products

Our present study showed that the editing properties of an enzyme are not frozen but can vary considerably according to the compound to be edited. With Nva, hcLeuRS exhibited a clear preference for post-transfer editing. However, with ABA, the enzyme used mainly pre-transfer editing. These different preferences for editing non-cognate compounds highlighted the modular character of the editing pathways of aaRS.

Pre- and post-transfer editing reactions are usually considered as the clearing mechanisms for the amino acid activation and aminoacylation reactions, respectively. We can reasonably assume that a misactivated compound can be edited by the pre-transfer mechanism or released from the enzyme (kinetic proofreading) first. The non-cognate activated amino acid can also be charged to produce misacylated-tRNA and be deacylated in a special editing domain by the post-transfer pathway. Therefore, the preference given by an enzyme to a pathway would directly depend on the capacity of the non-cognate amino acids to be activated and charged, and its capacity to be eliminated at different editing checkpoints (19,28,30,67–69). Pre-steady state kinetics performed on class I Ile- and ValRS and class II ThrRS have recently confirmed that the contribution of pre- and post-transfer editing are subjected to modulation by the rate of aminoacyl transfer (70), leading to the concept of kinetic partitioning between the two pathways (71).

Therefore, the use of pre-steady state kinetics with different non-cognate amino acids may help to establish which of the different editing pathways is the preferred pathway (70).

The post-transfer editing mechanism is relatively well known and accepted, while the different pre-transfer editing mechanisms are actively discussed. The precise location(s) of the pre-transfer editing site and the function(s) of tRNA are uncertain, oscillating between the sites located on CP1 editing domain (15,48,72–74) or on the synthetic domain (24,31,75). Herein, the AMP formation rates of the native hcLeuRS in the presence of AN2690 and tRNA showed that a tRNA-dependent pre-transfer editing reaction still exists when the tRNA is blocked in an inactive conformation into the CP1 editing site. This result suggests that the tRNA-dependent pre-transfer editing reaction occurs more likely into the synthetic site as the CP1 editing site is bound by AN2690. The editing properties of mutant D399A also support this conclusion. Although the post-transfer editing of the mutant was lost, the presence of tRNA stimulated ABA editing as expected from the tRNA-dependent pre-transfer editing reaction. Altogether, these data strongly suggest that the synthetic active site also contains the editing site where the tRNA-dependent pre-transfer editing catalysis occurs.

In conclusion, our data suggest that pre-transfer editing preferentially targets some non-cognate amino acids, whereas post-transfer editing is more efficient with other structurally related non-cognate amino acids. The preference could result from the capacity of the non-cognate amino acids to be charged onto the tRNA. This might explain that hcLeuRS favored post-transfer editing with Nva and pre-transfer editing with ABA. We are currently studying the editing properties of hcLeuRS for various non-cognate amino acids in order to confirm this result.

#### SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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